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Contribution of Salt Bridges Toward Protein Thermostability

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Sandeep Kumar[1], Chung-Jung Tsai[2], Buyong Ma[2] and Ruth Nussinov[1,3]*

[1]Intramural Research Support Program,
SAIC Frederick National Cancer Institute,
Cancer Research
and Development Center,
Laboratory of Experimental
and Computational Biology,
Bldg 469, Rm 151,
Frederick, MD 21702
[2]Laboratory of Experimental
and Computational Biology,
NCI-FCRDC,
Bldg 469, Rm 151,
Frederick, MD 21702
[3]Sackler Institute of Molecular Medicine,
Sackler School of Medicine,
Tel Aviv University,
Tel Aviv 69978, Israel

*Phone: 301-846-5579;
Fax: 301-846-5598;
E-mail: ruthn@ncifcrf.gov

Abstract

We present an extensive study of the structural factors suggested to be responsible for thermostability, in 18 nonredundant families of thermophilic and mesophilic proteins. Each of these 18 families consists of homologous thermophile-mesophile pairs, with high resolution crystal structures for both pair-members available in the Protein Data Bank (PDB). We observe that both the thermophilic and the mesophilic proteins have similar hydrophobicities, oligomeric states, and hydrogen bonds. On the other hand, salt bridges increase in most of the thermophilic proteins. Yet, on the other hand, salt bridges have been proposed to destabilize protein structures. Hence, here we seek to understand why do salt bridges occur more frequently in thermophilic proteins. Investigating this problem, we focus on the glutamate dehydrogenase family. Computation of the electrostatic contribution of salt bridge energies by solving the Poisson equation in a continuum solvent medium, shows that the salt bridges in the glutamate dehydrogenase from the hyperthermophile *Pyrococcus furiosus* are highly stabilizing. In contrast, the salt bridges in the mesophilic *Clostridium symbiosum* glutamate dehydrogenase contribute only marginally to protein stability. The presence of a larger number of salt bridges cooperatively enhances their strength. Our results indicate that salt bridges and their networks may have an important role in *rigidifying* the protein structure at high temperatures. Formation of salt bridge networks may help in explaining the increased occurrence and stability of salt bridges in hyperthermophiles.

Introduction

There has been a growing interest in understanding the stabilization of proteins from extremophiles. Such an understanding, especially of the thermophilic proteins, is critical for designing efficient enzymes for such applications as detergent manufacturing, food and starch processing, production of high fructose corn syrup and PCR (1). Innumerable investigators have focused on the problem of the molecular basis of protein thermostability. Several reasons have been attributed to the greater stability of the thermophilic proteins (2), including greater hydrophobicity (3), better packing, deletion or shortening of loops (4), smaller and less numerous cavities, increased surface area buried upon oligomerization (5), amino acid substitutions within and outside the secondary structures (3,4,6), increased occurrence of proline residues (3,7,8), decreased occurrence of thermolabile residues (4), increased helical content, increased polar surface area (3,9,10), better hydrogen bonding (9-11) and better salt bridges (3,4,11-13).

It is interesting to note that while on the protein level several differences have been observed between the thermophiles and the mesophiles, so far that has not been the case genome-wise. Complete genomes have been sequenced for a few hyperthermophilic organisms (14-16). However, inspection of the DNA sequences *per se*, so far did not yield any clues.

We have analyzed eighteen nonredundant families which contain pairs of high resolution structures of proteins from both thermophilic and mesophilic organisms. We

observe that both the thermophilic and their mesophilic protein counterparts have similar hydrophobicities, oligomeric states, main chain-main chain and main chain-side chain hydrogen bonds. On the other hand, the number of salt bridges increases for several thermophilic proteins (17).

Salt bridges have been suggested to be stabilizing (18-22), insignificant (23-25), or destabilizing (26-29) to the protein structure. Honig and Hubbell (30) estimated that the cost of transferring a salt bridge from water to the protein environment is approximately 10 - 16 kcal/mol. This large desolvation penalty is generally not recovered by favorable interactions in the folded/bound states (28,29). Hence, if salt bridges are destabilizing, why do they occur with higher frequencies in thermophilic proteins? How can the two facts be reconciled with each other?

Studies on the effects of temperature on protein hydration and the estimates of the free energies of folding of the proteins indicate a reduced desolvation penalty for salt bridges at high temperatures. This can be one of the reasons for the observed increase in the number of salt bridges in the hyperthermophiles (31). Here we present results of our calculations of the free energy changes upon salt bridge formation for one of the 18 families. We compare the values obtained for the thermophilic member with the mesophilic one.

Pyrococcus furiosus glutamate dehydrogenase (PfGDH) is extremely thermostable, with a half life of 12 hours at 100°C (32). Its melting temperature (T_m) is 113°C (33). The mesophilic *Clostridium symbiosum* glutamate dehydrogenase (CsGDH) shares 34% sequence identity with PfGDH. In both organisms, biochemically active GDH is a homohexamer. 3D structures for both GDHs are available (12,34,35) and are highly similar. In contrast to PfGDH, CsGDH has a half life of only 20 minutes at 52°C (12). Its melting temperature (T_m) is 55°C (13). The major difference between the two proteins is that the thermostable molecule has a substantially larger number of salt bridges, frequently arranged in extensive networks (12,13). We have performed continuum electrostatics calculations to estimate and compare salt bridge strengths in PfGDH and CsGDH. We find that salt bridges are highly stabilizing for the thermophilic glutamate dehydrogenase (PfGDH). In contrast, they contribute only marginally toward the stability of the mesophilic glutamate dehydrogenase (CsGDH). Salt bridges and their networks rigidify protein structures. A higher concentration of salt bridges, particularly networks, "stitches" the structure, making it more resistant to local deformation/melting or unfolding at high temperatures. Engineering networks of salt bridges in a mesophilic protein is expected to cooperatively enhance its thermal stability.

Materials and Methods

Families of Thermophilic and Mesophilic Proteins

We have searched the Protein Data Bank (PDB) (36) for the thermophilic proteins. For each of the thermophilic proteins, the PDB entry with the best resolution was picked. The 3D structures of thermophilic proteins were compared all against all using a sequence order independent structural comparison technique (37). Two proteins were considered to be dissimilar if:

(i) Backbone C^{α} atom superposition for the two structures yields root mean square deviation (r.m.s.d.) ≥ 2.00Å,

and

(ii) Sequence identity for two proteins (ID) ≤ 20%.

Finally, structurally nonhomologous thermophilic proteins were retained if there was at least one high resolution crystal structure for their corresponding mesophilic homologues. In this way, 18 nonredundant families were constructed for the thermophilic-mesophilic proteins.These families are: Citrate synthase (1AJ8 and 1CSH), malate dehydrogenase (1BDM and 4MDH), rubredoxin (1CAA and 8RXN), cyclodextrin glucanotransferase (1CIU and 1CDG), EF-TU and EF-TU/TS complex (1EFT and 1EFU), EF-TS and EF-TU/TS complex (1TFE and 1EFU), glutamate dehydrogenase (1GTM and 1HRD), lactate dehydrogenase (1LDN and 1LDG), thermolysin and neutral protease (1LNF and 1NPC), 3-phosphoglycerate kinase (1PHP and 1QPG), CheY (1TMY and 3CHY), methionine aminopeptidase (1XGS and 1MAT), Xylanase (1YNA and 1XNB), adenylate kinase (1ZIN and 1AKY), ferredoxin (2FXB and 1FCA), inorganic pyrophosphatase (2PRD and 1INO), manganese superoxide dismutase (3MDS and 1QNM), and phosphofructokinase (3PFK and 2PFK).

The corresponding thermophilic and mesophilic proteins within these families are highly similar, with sequence identities varying over a range of 24 - 73% and backbone root mean square deviations (r.m.s.d.) being 0.69 - 1.68Å. At the same time, the thermophilic proteins across the 18 families are highly dissimilar among themselves (sequence identities being <10% and backbone r.m.s.d. >2Å). The mesophilic proteins are also highly dissimilar among themselves.

Database of 165 Dissimilar Monomers

A database of 165 proteins, which (i) have been solved to high resolution R ≤2.5Å by X-ray crystallography and contain at least 50 amino acids, (ii) have dissimilar three dimensional structures, as determined by the sequence order independent structure comparison technique (37), and (iii) exist as monomers in solution as indicated in their PDB files and relevant biochemical literature has been constructed.

Structural Properties Compared Between Thermophilic and Mesophilic Proteins

(i) Oligomeric State

The biochemically relevant oligomeric states of the thermophilic and mesophilic proteins were obtained by studying the biochemical data contained in the relevant literature on these proteins.

ii) Hydrophobicity

The hydrophobicity of a protein was calculated as the fraction of the buried non-polar area out of the total non-polar area, computed by using the methods described earlier (38-40).

(iii) Hydrogen Bonds and Salt Bridges

Whenever two heavy (non-hydrogen) atoms with opposite partial charges (Donor (D) - Accepter (A) pairs) were found to be within a distance of 3.5Å, a hydrogen bond has been inferred. The geometrical goodness of the hydrogen bond was assessed by computing the values of following angles:

Angle θ_D between vectors BD - D and D - A, BD is the atom covalently bonded to the donor (D) atom.

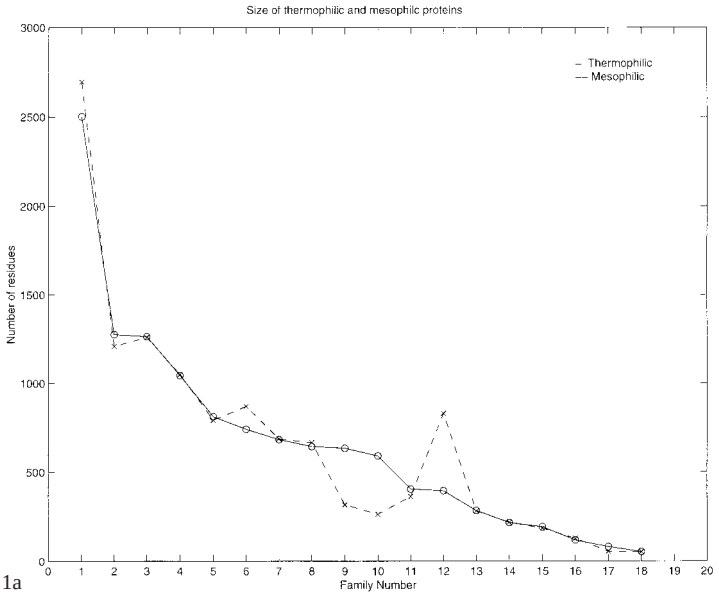
Angle θ_A between vectors D - A and A - BA, BA is the atom covalently bonded to the acceptor (A) atom.

The presence of salt bridges was inferred when Asp or Glu side chain carbonyl oxygen atoms were found to be within 4.0Å distance from the nitrogen atoms in Arg, Lys and His side chains.

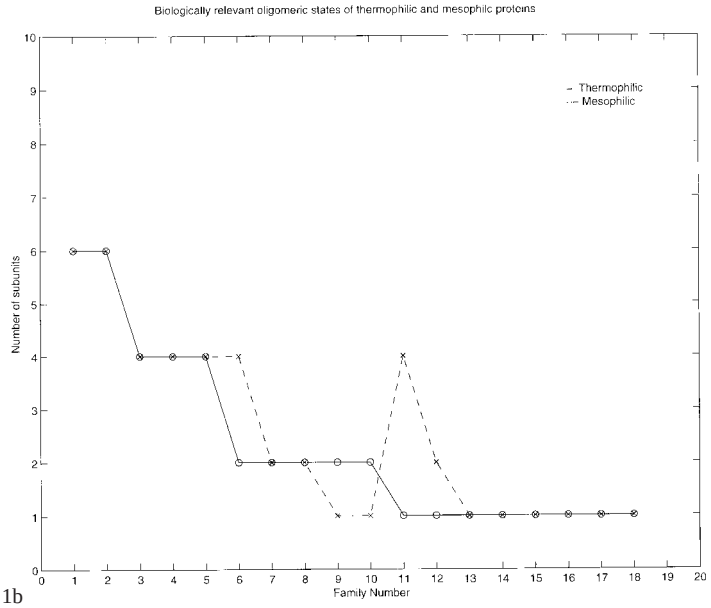
The location of residues forming salt bridges was characterized in terms of their solvent accessible area (ASA) (38,39,41) with a probe radius of 1.4Å. A residue X was classified as being exposed if its ASA is above 20% of the ASA calculated for tripeptide GLY - X - GLY in an extended conformation. Otherwise, it was classified as being an internal residue (40). The location of the salt bridge in the protein was assessed by the average of percent ASAs of the individual residues.

Computation of Electrostatic Energies of Salt Bridges

Continuum electrostatic calculations were performed with the DELPHI package (42-46) under INSIGHTII release 98.0. PARSE3 set of partial atomic charges and atomic radii (47) were used. The PARSE set allows reproduction of the experimental data for a wide range of small organic molecules and ions representing side chains of amino acids (48). The solvent probe radius used to define the molecular surface was 1.4Å. A 2Å Stern layer (49) was applied to exclude ions from the molecular surface. The dielectric constant for the solute (protein molecule) was 4.0 and that for solvent was 80.0. The ionic strength was 0.0 M. The Poisson equation was solved using the iterative finite difference method (43-45) on a 3 dimensional grid with step of 0.833Å per grid point, with an energy convergence criterion of



1a



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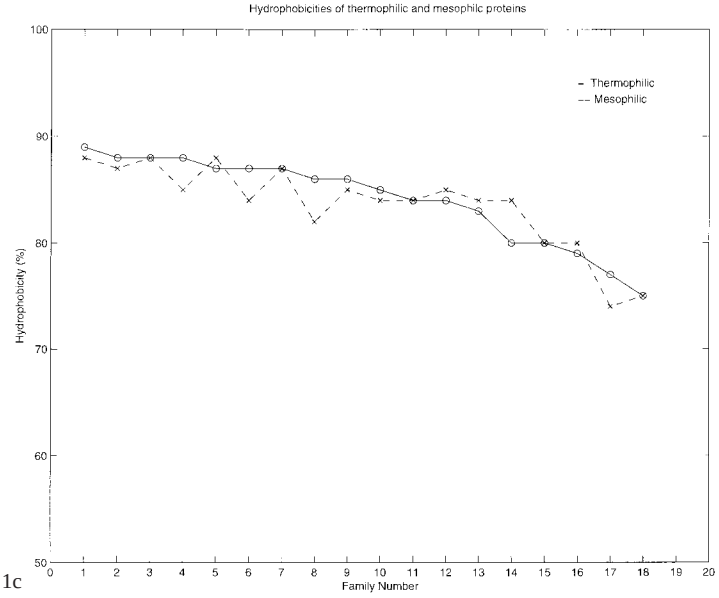

1c

Figure 1: Comparison of various structural properties in thermophilic and mesophilic proteins. (a) Size of the proteins. X-axis represents the family number and Y-axis represents number of residues in the biologically active state of thermophilic and mesophilic proteins. (b) Oligomeric states. X-axis represents the family number and Y-axis represents the number of subunits in the biologically active states of the thermophilic and mesophilic proteins. (c) Hydrophobicities. X-axis represents the family number and Y-axis represents the hydrophobicity of the thermophilic and mesophilic proteins in their biologically active states. It can be seen from this figure that there are no consistent differences between thermophiles and mesophiles for these proteins.

1×10^{-6} kT (kT are the units of energy in DELPHI outputs, k is Boltzmann's constant and T is absolute Temperature).

In each calculation, at first the molecule occupied 50% of the grid and Debye-Huckel (Full Coulombic) boundary conditions were applied (50). The resulting grid of this rough calculation was used as boundary condition for a focused calculation in which the molecule occupied 95% of the grid. The results of the focused calculation are presented here. The dielectric constant of water at 100°C is 55.51 (51). The DELPHI calculations were also performed with solvent dielectric constant of 55.51 for PfGDH.

Results and Discussion

Structural Properties

Figure 1 (a), (b), and (c) compare the number of residues, oligomeric states and hydrophobicities of the thermophilic and mesophilic proteins in the eighteen families, respectively. These properties do not show any consistent differences between the thermophilic and mesophilic proteins. Table I presents the results of various fractional surface areas for thermophilic and mesophilic proteins chains along with those for a set of 165 non-homologous proteins with dissimilar structures. The distributions of polar and nonpolar surface area are similar between thermophilic and mesophilic protein chains and fall within the range expected from the 165 dissimilar monomers.

Hydrogen bonds and salt bridges have also been compared between the thermophilic and the mesophilic proteins. Salt bridge content increases in thermophilic proteins for 13 out of 18 families. Hence, salt bridges are the only structural feature that show consistent increase with thermal stabilities of the proteins in our database. To further probe the contribution of salt bridges in thermophilic proteins, we focus on the family with the maximum increase in the number of salt bridges. An earlier analysis of glutamate dehydrogenase has indicated that the increased number of salt bridges and their networks is responsible for the greater stability of the thermophilic glutamate dehydrogenasm (PfGDH) "versus" its mesophilic counterpart (CsGDH) (12).

PfGDH and CsGDH Salt Bridges

Biochemically active hexamers of PfGDH and CsGDH contain 168 and 107 salt bridges respectively. This amounts to a ~ 70% increase in the frequency of salt bridges in PfGDH over CsGDH (normalizing by the number of residues in PfGDH and CsGDH). 128 (76.2%) out of the 168 salt bridges in the PfGDH are formed within the six subunits and 40 (23.8%) salt bridges are formed across the subunit interfaces. In comparison, 75 (70.1%) out of 107 salt bridges in CsGDH are formed within the subunits and 32 (29.9%) salt bridges are formed across the subunit interfaces.

The B chain of 1GTM contains 40 salt bridges, while that of 1HRD contains only 20 salt bridges. Most of the salt bridges in the GDHs are either partially exposed (one of the salt bridge forming residues has >20% ASA as compared to the extended conformation, and the other has <20% ASA) or are on the protein surface (both the salt bridge forming residues have >20% ASA). Furthermore, the B chain of 1GTM contains 8 salt bridge networks, 2 triads, 3 tetrads, 2 pentads and 1 hexad. In contrast, the B chain of 1HRD contains only two triads and a tetrad.

Electrostatic Energies of Salt Bridges in Monomers of PfGDH and CsGDH

The electrostatic energy of a salt bridge, $\Delta\Delta G_{tot}$, was calculated by the following equation:

$$\Delta\Delta G_{tot} = \Delta\Delta G_{dslv} + \Delta\Delta G_{brd} + \Delta\Delta G_{prt}$$

where

$\Delta\Delta G_{dslv}$ is an unfavorable desolvation free energy penalty (desolvation penalty) incurred due to desolvation of salt bridge forming side chains.

$\Delta\Delta G_{brd}$ is energy of the interaction between twosalt bridge

Table I

Various fractional surface areas.

Averages of various fractional surface areas in thermophilic, mesophilic protein chains and 165 monomers with dissimilar structures. Only one chain per protein was considered in these calculations.

1. Fractional polar exposed area (FPEA) is the ratio of polar exposed surface area to total exposed surface area.
2. Fractional nonpolar exposed area (FNPEA) is the ratio of nonpolar exposed surface area to total exposed surface area.
3. Fractional polar buried area (FPBA) is the ratio of polar buried surface area to total buried surface area.
4. Fractional nonpolar buried area (FNPBA) is the ratio of nonpolar buried surface area to total buried surface area.

Note that FPEA+FNPEA=1. Similarly, FPBA+FNPBA=1.

5. Fractional nonpolar area (FNP) is the ratio of nonpolar buried surface area to total nonpolar area. This ratio is same as hydrophobicity defined in materials and methods section.
6. Fractional polar surface area (FP) is the ratio of polar exposed surface area to total polar area.

Fractional surface area	165 monomers	Thermophilic protein chains	Mesophilic protein chains
FPEA[1]	0.493 ± 0.036	0.489 ± 0.027	0.482 ± 0.031
FNPEA[2]	0.507 ± 0.036	0.511 ± 0.027	0.518 ± 0.031
FPBA[3]	0.568 ± 0.014	0.557 ± 0.011	0.565 ± 0.013
FNPBA[4]	0.432 ± 0.014	0.443 ± 0.011	0.435 ± 0.013
FNP[5]	0.816 ± 0.039	0.824 ± 0.034	0.817 ± 0.034
FP[6]	0.142 ± 0.026	0.139 ± 0.022	0.138 ± 0.025

Table IIa and **IIb**

Comparison of electrostatic strengths of salt bridges in thermophilic and mesopphilic glutamate dehydrogenase, PfGDH and CsGDH. ASA_{av} indicates average accessible surface area of the two residues forming a salt bridge in the hexameric state of glutamate dehydrogenase. $\Delta\Delta G_{tot}$ refers to the total electrostatic free energy of the salt bridge.$\Delta\Delta G_{dslv}$ indicates the desolvation energy penalty incurred by the salt bridge. $\Delta\Delta G_{brd}$ is the free energy of the interaction of salt bridge forming side chains with each other.$\Delta\Delta G_{prt}$ is the free energy of the interaction of salt bridge forming side chains with the rest of the protein. Complete description of the energy terms is given in the text. Energies are presented for only those salt bridges which have equivalent locations in the trimeric (crystal asymmetric unit) and hexameric (functional form) states of glutamate dehydrogenase.

Table IIa

Energies of salt bridges in CsGDH (B chain of 1HRD).

Salt Bridge	ASA_{av} (%)	$\Delta\Delta G_{tot}$ (Kcal/mole)	$\Delta\Delta G_{dslv}$ (Kcal/mole)	$\Delta\Delta G_{brd}$ (Kcal/mole)	$\Delta\Delta G_{prt}$ (Kcal/mole)
R6 - E10	42.8	+0.095	2.870	-1.095	-1.681
R6 - E43	40.4	-1.099	3.039	-1.979	-2.159
E18 - K104	7.5	+1.455	10.332	-7.060	-1.818
H39 - E41	25.6	-1.333	5.838	-2.796	-4.377
R78 - D160	0.0	-6.331	25.348	-27.274	-4.405
R93 ± D165	25.9	-7.006	6.581	-9.573	-4.014
K125 ± D165	18.3	-6.024	9.213	-4.964	-10.273
D137 ± R171	45.7	-0.299	4.743	-2.195	-2.847
R171 ± E172	22.4	-6.830	6.836	-6.006	-7.660
E218 ± H403	22.4	+1.445	4.198	-3.638	+0.454
E224 ± K340	25.3	+3.422	5.456	-2.498	+0.463
D226 ± K231	24.7	-6.303	4.887	-5.142	-6.049
K248 ± E251	34.4	+0.800	5.813	-2.267	-2.747
D268 ± K277	4.7	-1.555	15.187	-13.456	-3.288
E276 ± K298	52.7	-1.558	1.402	-1.714	-1.246
R289 - D294	33.6	-0.342	7.462	-2.473	-5.332
H410 - D411	24.5	+6.796	9.316	-3.933	+1.413
Average	26.5 ± 14.3	-1.476 ± 3.910	7.560 ± 5.628	-5.768 ± 6.398	-2.627 ± 2.497
Average (37°C)	26.5 ± 14.3	-1.536 ± 4.068	7.867 ± 5.856	-6.002 ± 6.657	-2.734 ± 2.598

Table IIb

Energies of salt bridges in PfGDH (B chain of 1GTM).

Salt Bridge	ASA_{av} (%)	$\Delta\Delta G_{tot}$ (Kcal/mole)	$\Delta\Delta G_{dslv}$ (Kcal/mole)	$\Delta\Delta G_{brd}$ (Kcal/mole)	$\Delta\Delta G_{prt}$ (Kcal/mole)
E7 - K11	50.6	+1.913	3.172	-1.032	-0.226
R15 - D397	20.8	-4.898	5.832	-7.833	-2.898
R57 - D139	0.0	-5.878	25.646	-27.240	-4.284
R72 - E77	7.2	-7.135	12.917	-18.260	-1.792
R72 - D144	22.9	-15.258	10.164	-3.737	-21.684
D97 - K379	38.1	-1.965	4.511	-3.589	-2.886
K102 - D144	19.3	-3.355	7.718	-5.302	-5.771
E188 - R192	7.1	-12.053	7.961	-10.757	-9.257
E188 - R370	19.2	-10.471	7.663	-11.604	-6.532
R192 - D234	32.0	-1.106	7.126	-2.354	-5.878
R199 - E200	10.8	-6.479	10.418	-7.128	-9.768
R199 - D374	21.4	-3.747	7.660	-7.595	-3.773
E200 - K203	27.0	-4.797	6.919	-6.745	-4.970
D208 - K213	52.6	-4.206	4.122	-4.821	-3.508
K229 - E233	41.4	-8.023	3.955	-7.857	-4.120
K229 - D258	25.7	-4.735	6.301	-4.565	-6.471
D244 - K264	13.0	-5.016	13.532	-5.572	-12.975
E259 - K262	47.5	-1.575	0.086	-1.677	+0.015
K271 - D272	48.1	-3.623	0.736	-4.329	-0.030
D290 - K312	31.2	-1.549	5.005	-2.139	-4.415
D307 - K333	50.2	-2.958	2.124	-1.846	-3.236
E316 - R396	4.4	-5.376	16.371	-12.855	-8.893
D327 - H394	6.5	+8.667	14.091	-5.362	-0.062
D327 - R396	7.6	-8.418	12.442	-9.235	-11.627
D340 - R396	5.3	-11.077	13.007	-3.220	-20.865
E371 - K375	54.0	-0.873	1.784	-2.028	-0.629
K379 - D383	32.3	-1.642	5.858	-3.792	-3.708
D383 - R406	19.4	-1.204	7.495	-5.039	-3.661
E390 - K391	55. 6	-1.432	0.903	-2.019	-0.316
Average	26.6 ± 17.5	-4.413 ± 4.591	7.774 ± 5.535	-6.521 ± 5.574	-5.667 ± 5.526
Average (100°C, ε = 55.51)	26.6 ± 17.5	-6.488 ± 5.985	9.931 ± 6.618	-9.024 ± 6.962	-7.395 ± 7.121

forming residue side chains.

$\Delta\Delta G_{prt}$ represents the free energy of the interaction of salt bridgeforming side chains with the rest of the protein.

Continuum electrostatics analyses of salt bridge formation in the B subunits of 1GTM (PfGDH) and 1HRD (CsGDH) were performed using the DELPHI package. We have used only the trimers (as in the crystal asymmetric unit) in our calculations due to a limitation of the available computing power. This simplification faces the danger of underestimating the desolvation penalty in the case of salt bridges at or near the interfaces. To work around this difficulty, we have made use of the accessible surface area (ASA) (38,39,41) to estimate the location of salt bridge forming residues in the trimeric and hexameric states of glutamate dehydrogenase. If both residues in the salt bridge have similar ASAs in the trimeric and the hexameric states, it is assumed that the environment of the salt bridge is similar between the trimer and the hexamer. This implies that the estimates of the desolvation energy penalty obtained by our calculations are reasonably accurate. Residue pairs 29 out of the 40 salt bridges in chain B of 1GTM have similar ASAs in the trimeric and hexameric states. In the case of CsGDH, chain B of 1HRD has 17 salt bridges where both residues have nearly identical ASAs in both states.

The results of the salt bridge calculations for CsGDH and PfGDH are presented in Tables II(a) and (b). The thermophilic protein (1GTM) has only 2 out of 29 (6.9%) salt bridges which are destabilizing. On the other hand, the mesophilic protein (1HRD) has 6 out of 17 (35.3%) salt bridges which are destabilizing. The unfavorable desolvation energy penalty is compensated by the bridge and protein energy terms by only a small margin in the salt bridges of the mesophilic protein (1HRD) (average total free energy change = -1.476 kcal/mol). In contrast, average desolvation penalty is more than compensated by the bridge and protein energy terms in the salt bridges of the thermophilic protein (1GTM) (average total free energy change = -4.413 kcal/mol).

The temperatures of optimum growth for the thermophile and mesophile differ from the room temperature. Furthermore, the dielectric constant of water decreases to 55.51 at 100°C (51), the optimum growth temperature for *Pyrococcus furiousus*. Recently, it has been shown that hydration free energies of amino acid residues change with temperature, due to a decrease in the dielectric constant of water and an increase in the atomic radii with an increase in temperature (52). The calculated and experimental changes in the hydration free energies of amino acids are typically on the order of 1 Kcal/mol for an increase in temperature from 25°C to 100°C (52). Tables II(a) and (b) present the average values for the salt bridge energy terms at the respective growth temperatures of the mesophilic and thermophilic organisms. The salt bridges in the mesophile have similar energies at their optimum growth temperature and at room temperature. In contrast, the thermophilic salt bridges become stronger, with the average free energy change decreasing from -4.41 Kcal/mol to -6.49 Kcal/mol, when the appropriate corrections are applied.

What causes salt bridges to be highly stabilizing in PfGDH, in contrast to its counterpart, CsGDH? Salt bridge networks and the cooperative nature of electrostatic interactions may provide a clue. Table II(a) shows that for the mesophilic glutamate dehydrogenase (CsGDH), the interaction energy between the charged side chains in the salt bridges ($\Delta\Delta G_{brd}$) is considerably larger in magnitude than the interaction energy between the charged side chains and the rest of the protein ($\Delta\Delta G_{prt}$). On the other hand, $\Delta\Delta G_{brd}$ and $\Delta\Delta G_{prt}$ have similar magnitudes in PfGDH. On average, the magnitude of $\Delta\Delta G_{prt}$ is approximately double in PfGDH salt bridges as compared to that in CsGDH salt bridges. The average $\Delta\Delta G_{prt}$ in PfGDH is -5.667 Kcal/mol and that in CsGDH is -2.627 Kcal/mol. PfGDH is particularly rich in salt bridge networks (12,13). There are eight clusters of salt bridge networks in the B chain of 1GTM. In total, these clusters account for 23 out of the 29 salt bridges studied here. Our calculations indicate that 6 of these 23 salt bridges are highly stabilizing. In these salt bridges, the protein energy terms have large magnitudes. Thus, extensive networks of salt bridges are particularly favorable, by cooperatively contributing to stabilize the protein. During protein folding, the cooperative nature of salt bridges and their networks is favorable kinetically, guiding correct folding and limiting the number of alternate allowed folded conformations. This cooperativity can resist unfolding, opposing disorder due to atom mobility. This is particularly crucial at high temperatures. Thus, salt bridge networks may provide a mechanism to counteract melting/unfolding.

In summary, our results suggest an explanation for the increased frequency of salt bridges in PfGDH as compared to CsGDH. These also indicate the possible origin ofthe increased stability of the salt bridges and the advantages gained by formation of salt bridge networks in the PfGDH thermophile.

Conclusions

Comparison of high resolution crystal structures of thermophilic and mesophilic proteins can be a very useful tool for investigations of thermostability. Among all the structural parameters which we have studied, only salt bridges increase consistently in the thermophilic proteins. While other structural factors, *i.e.*, oligomeric state, hydrophobic cores and hydrogen bonds contribute to protein thermostability, their contribution appears to be more variable. The question that immediately comes to mind is, why are there more salt bridges in thermophiles? This question is particularly pertinent, given the observation made by Hendsch and Tidor (28) that salt bridges are destabilizing. We have explored this question using glutamate dehydrogenase from thermophilic and mesophilic sources. We observe that, on average, salt bridges are highly stabilizing in the thermophilic glutamate dehydrogenase. In contrast, on average, they are only marginally stabilizing in its mesophilic counterpart. It appears that the origin of much of the stabilizing electrostatic contribu-

tion derives from $\Delta\Delta G_{prt}$, the protein neighborhood of the salt bridge. The larger number of charged residues, and of bridges around a particular bridge, as in the case of the thermophilic protein, enhance the stability of the bridge. This is in agreement with the commonly held view that nearby salt bridges cooperatively and mutually strengthen each other.

Furthermore, isolated, and networks of salt bridges can provide kinetic barriers against local, and nonlocal deformation in protein structures at high temperatures. Salt bridges can stitch and *rigidify* protein structures. Engineering several close by single, and networks of salt bridges in a mesophilic protein may substantially enhance its thermal stability.

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